Atty. Dkt. No. 355908-1300

*IN THE UNITED STATES PATENT AND TRADEMARK OFFICE*

Applicant: Pavel HAMET et al.

Title: PRE-CONDITIONING CELLS AGAINST CELL DEATH

Appl. No.: 09/480,260

Filing Date: January 11, 2000

Examiner: M. Meller

Art Unit: 1654

DECLARATION UNDER 37 C.F.R. §1.132

I, **Marina A. Lynch**, Ph.D., hereby declare that:

1. I am Lecturer and Associate Professor at Trinity College, Dublin, Ireland. I have authored or co-authored over 100 published scientific papers in the field of synaptic plasticity over the past 20 years. A copy of my Curriculum Vitae is attached hereto as **Exhibit A**.

2. I am a paid consultant to Vasogen Inc. (Mississauga, Canada) and Vasogen Ireland Ltd. (Shannon, Co. Clare, Ireland) (collectively, "Vasogen"). I have conducted studies funded by Vasogen in the area of assessment of the effects of certain of Vasogen's therapeutic compositions on synaptic activity in rat hippocampus.

3. I am a co-author of the research paper entitled "Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy," by Y. Nolan, A. Minogue, E. Vereker, A. E. Bolton, V.A. Campbell and M.A. Lynch, published in the journal *Neuroimmunomodulation*, volume 10, pages 40-46 (2002-2003), (hereinafter, the "Nolan Paper"). A copy of this paper is attached hereto as **Exhibit B**.

4. I have reviewed the subject U.S. Patent Application Serial Number 09/480,260 (hereinafter, the "Hamet Application") and the U.S. Patent Examiner's remarks concerning enablement of claims directed to methods of alleviating or protecting against the symptoms of a medical disorder involving accelerated rates of apoptosis.

5. The Hamet Application describes a method of reducing a mammalian subject's susceptibity to a condition characterized by apoptotic death of cells, by administering to the

subject an aliquot of blood that has been subjected to one or more of certain stressor condition(s) ("IMT Treatment"). The results reported in the Hamet Application indicate that animals who receive such treatment exhibit lower levels of apoptotic cell death in areas subjected to apoptosis-inducing events.

6. I have studied the phenomenon of apoptotic cell death in an experimental model of brain cell function, the electrophysiological measurement of long term potentiation (LTP) in the hippocampus of the anaesthetized rat. This preparation is exquisitely sensitive to systemic administration of lipopolysaccharide (LPS), an inducer of pro-inflammatory cytokines. As shown in the Nolan Paper, peripheral administration of LPS resulted in increased levels of the inflammatory cytokine IL-1 (*C.f.,* Figure 2, page 43), increased apoptotic cell death, as measured by TUNEL staining (*C.f.*, Figure 5, page 45), as well as decreased synaptic function, as evidenced by reduced LTP activity in LPS-treated animals, as compared to control animals (Figure 1, page 4).

7. In experiments reported in the Nolan Paper, my co-authors and I demonstrated that pretreatment of rats with Vasogen's IMT Treatment prevented most if not all of the above negative effects. Specifically, the IMT Treatment inhibited LPS-induced apoptotic changes in brain hippocampal cells (*C.f.*, Figure 5, page 45). Furthermore, the IMT Treatment is a blood stressor treatment that falls within the description of the treatment modality claimed in the Hamet Application.

8. On the basis of our studies, we concluded that "...pretreatment with the [IMT Treatment] confers a protective effect ... by preventing LPS-induced impairment of synaptic function and the resultant detrimental effects in the hippocampus of the rat."(page 46, last paragraph).

9. We therefore showed that Vasogen's IMT Treatment, which is the treatment claimed in the Hamet Application, confers a protective effect on susceptible neuronal cells.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at TCD Dublin, Ireland, this 26 day of August, 2004.

Marina A. Lynch

# CURRICULUM VITAE: Marina A Lynch

**Name:** Marina Annetta LYNCH
**Date of Birth:** 10th March, 1953
**Address:** Department of Physiology, Trinity College, Dublin 2
**Qualifications:** B.Sc. (NUI), MSc. (NUI), PhD (Dubl)

**Positions Held**

| | |
|---|---|
| 1999-Present | Associate Professor of Physiology, University of Dublin |
| 1992-1998 | Lecturer, Department of Physiology, University of Dublin |
| 1988-1992 | Research Scientist, National Institute for Medical Research, London |
| 1983-1988 | Postdoctoral Fellow, National Institute for Medical Research, London |
| 1981-1983 | Postdoctoral Fellow, King's College, London |

**Institutional Responsibilities include**

University of Dublin Academic Affairs Committee; Director of Postgraduate Studies Trinity College Institute of Neuroscience; University of Dublin Life Sciences Strategic Planning Group; University of Dublin Disciplinary Panel

**External Responsibilities include**

**Member**: Commission to advise Government on Assisted Human Reproduction; Health Research Board Neuroscience Grants Committee

**Reviewer**: Wellcome Trust, Science Foundation of Ireland (Chair of Neuroscience panel 2004), Health Research Board, and various learned journals including J Neuroscience, Neuroscience, European J Neuroscience, Neuropharmacology, J Neurochemistry

**Awards and Distinctions include**

MRC Postdoctoral Fellowship (1981-1988)
Honorary Lecturer, Royal Free Hospital School of Medicine, London (1990-1992)
Elected to Fellowship, Trinity College Dublin (1997)
Plenary lectureships include (most recent).

Hippocampal Research Conference, Grand Cayman (2000), Society for Neurosciences, New Orleans (2000), Neurobiology of interleukin-1 receptors (EU symposium), Biarritz (2000), European Society of Neurochemistry, Perugia (2001), Portugese Society of Neuroscience, Peniche (2001), American Aging Association, San Diego (2002), IBRO World Congress of Neuroscience, Prague (2003), PsychoNeuroImmunology Research Society, Titisee Germany (2004), Federation of European Neurosciences, Lisbon (2004). Currently 5 invitations to participate in international conferences in 2005

Invited chapters and reviews (most recent)

Progress in Neurobiology (1998), Reviews in Neurosciences (1998), Molecular Psychiatry (1998), Vitamins and Hormones (2000), Nutritional Neurosciences (2000), Diet – Brain connections: Impact on memory, mood, ageing and disease (2001), Physiological Reviews (2003)

**Publications**

>120 peer-reviewed research papers, 20 invited reviews and book chapters, 150 conference proceedings

## PEER-REVIEWED PAPERS

1. Lynch, M., Kenny, M. and Leonard, B.E. (1977) Changes in the catecholamine content in 4 regions of the rat brain following acute amphetamine treatment: antagonistic effect of chlorpromazine. I.C.R.S. Med. Sci., 5, 561.

2. Lynch, M., Kenny, M. and Leonard, B.E. (1977) Changes in behaviour in rats in the "open field" apparatus after chronic amphetamine administration: acute effects of 3 antidepressants. I.C.R.S. Med. Sci., 5, 571.

3. Lynch, M., Kenny, M. and Leonard, B.E. (1977) The effect of chronic administration of D-amphetamine on regional changes in catecholamines in the rat brain. J. Neurosci. Res., 3, 295.

4. Lynch, M. and Leonard, B.E. (1978) Changes in brain gamma aminobutyric acid concentrations following acute and chronic amphetamine administration and during post-amphetamine depression. Biochem. Pharmacol., 27, 1853.

5. Lynch, M., Kenny, M. and Leonard, B.E. (1978) Effect of D-amphetamine on the behaviour of rats in the "open field": interaction with 4 neuroleptics and changes in biogenic amine metabolism in discrete brain regions. Irish J. Med. Sci., 147, 337.

6. Lynch, M. and Leonard, B.E. (1978) Effect of chronic amphetamine administration on the behaviour of rats in the "open field" apparatus: reversal of post-withdrawal depression by 2 antidepressants. J. Pharm. Pharmac., 30, 798.

7. Kenny, M., Lynch, M. and Leonard, B.E. (1980) Induction of two distinct behavioural responses by chronic treatment with apomorphine. J. Neurosci. Res., 5, 35.

8. Fenn, G.C., Lynch, M., Nhamburo, P.T., Caberos, L.P. and Littleton, J.M. (1983) A comparison of effects of ethanol on platelet function and synaptic transmission. Pharmac. Biochem. Behav., 18 Suppl. 1, 37.

9. Samuel, D., Lynch, M. and Littleton, J.M. (1983) Interaction between ethanol and GABA on $^{3}H$ dopamine efflux from superfused slices of rat striatum. Neuropharmacology, 22, 1413.

10. Caberos, L.P., Lynch, M., Leroy, C., Fenn, G.C. and Littleton, J.M. (1983) Interaction of ethanol with ionophore A23187 in human platelets and erythrocytes and in rat brain slices. Biochem. Pharmacol., 32, 2211.

11. Lynch, M., Littleton, J.M., McKernan, R.M., Durcan, M.J., McMillan, J. and Campbell, I.C. (1983) Alpha-adrenoceptor number and function in rat cortex after ethanol and immobilization stress. Brain Res., 288, 145-149.

12. Lynch, M. and Littleton, J.M. (1983) Possible association of alcohol tolerance with increased synaptic $Ca^{2+}$ sensitivity. Nature, 303, 175-177.

13. Lynch, M., Andrews, J.F. and Moore, R.E. (1985) Administration of low doses of TSH result in a rapid increase in the metabolic rate of young lambs. Horm. Metabol. Res., 17, 136-140.

14. Lynch, M., Andrews, J.F. and Moore, R.E. (1985) Low doses of $T_3$ induce a rapid metabolic response in young lambs. Horm. Metabol. Res., 17, 63-66.

15. Lynch, M., Bruton, J.D., Andrews, J.F. and Moore, R.E. (1985) The rapid metabolic response of young lambs to low doses of $T_3$: interaction with $rT_3$. J. Therm. Biol., 10, 71-77.

16. Lynch, M., Samuel, D. and Littleton, J.M. (1985) Altered characteristics of [$^{3}H$] dopamine

release from superfused slices of corpus striatum obtained from rats receiving ethanol in vivo. Neuropharmacology, 24, 479-485.

17. Lynch, M. and Littleton, J.M. (1985) Enhanced $^{3}H$ noradrenaline release in synaptosomes from ethanol-tolerant animals: the role of nerve terminal $Ca^{2+}$. Alcohol & Alcoholism, 20, 5-11.

18. Lynch, M., Pagonis, C., Samuel, D. and Littleton, J.M. (1985) Alterations in $Ca^{2+}$-dependent and $Ca^{2+}$-independent release of catecholamines in preparations of rat brain produced by ethanol treatment in vivo. Alcohol, 2, 139-144.

19. Feasey, K.J., Lynch, M. and Bliss, T.V.P. (1985) Long-term potentiation is associated with an increase in calcium-dependent, potassium-stimulated release of $^{14}C$ glutamate from hippocampal slices: an ex vivo study in the rat. Brain Res., 364, 39-44.

20. Bliss, T.V.P., Douglas, R.M., Errington, M.L. and Lynch, M. (1986) Correlation between long-term potentiation and release of endogenous amino acids from dentate gyrus of anaesthetized rats. J. Physiol., 377, 391-408.

21. Lynch, M. and Bliss, T.V.P. (1986) Noradrenaline modulates release of $^{14}C$ glutamate from dentate but not from CA1/CA3 slices of rat hippocampus. Neuropharmacology, 25, 493-498.

22. Lynch, M., Errington, M.L. and Bliss, T.V.P. (1986) Long-term potentiation of synaptic transmission in dentate gyrus increased release of $^{14}C$ glutamate without an increase in receptor binding. Neurosci. Letts., 62, 123-129.

23. Lynch, M. and Bliss, T.V.P. (1986) On the mechanism of enhanced release of $^{14}C$ glutamate in hippocampal long-term potentiation. Brain Res., 369, 495-509.

24. Lynch, M., Archer, E.R. and Littleton, J.M. (1986) Increased sensitivity of transmitter release to calcium in ethanol tolerance. Biochem. Pharmacol., 3, 1207-1209.

25. Spencer, P., Lynch, M. and Bliss, T.V.P. (1986) In vitro release of $^{14}C$ glutamate from dentate gyrus in modulated by GABA. J. Pharm. Pharmacol., 38, 393-395.

26. Lynch, M.A. and Bliss, T.V.P. (1986) Long-term potentiation of synaptic transmission in CA3: effect of calmodulin and OAG on release of $^{3}H$ glutamate. Neurosci. Letts., 65, 171-176.

28. Errington, M.L., Lynch, M.A. and Bliss, T.V.P. (1987) Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(-)amino-phosphonovalerate. Neuroscience, 20, 279-284.

29. Bliss, T.V.P., Errington, M.L., Laroche, S. and Lynch, M.A. (1987) Increase in $K^{+}$-stimulated $Ca^{2+}$-dependent release of [$^{3}H$]-glutamate from dentate gyrus three days after the induction of long-term potentiation. Neurosci. Letts., 83, 107-112.

30. Laroche, S., Errington, M.L., Lynch, M.A. and Bliss, T.V.P. (1987) Increase in $^{3}H$ glutamate release from slices of dentate gyrus and hippocampus following classical conditioning in the rat. Behavioural Brain Res., 25, 23-29.

31. Lynch, M.A., Clements, M.P., Errington, M.L. and Bliss, T.V.P. (1988) Increases in hydrolysis of phosphatidylinositol (4,5) bisphosphate in long-term potentiation. Neurosci. Letts., 84, 291-296.

32. Clements, M.P., Lynch, M.A. and Bliss, T.V.P. (1988) The increase in phosphoinositide turnover associated with long-term potentiation may be mediated through a GTP binding protein. Neuroscience Research Communications, 3, 11-19.

33. Lynch, M.A., Errington, M.L. and Bliss, T.V.P. (1989) Nordihydroguaiaretic acid blocks the synaptic component of long-term potentiation and the associated increases in release of glutamate and arachidonate: an in vivo study in the dentate gyrus of the rat. Neuroscience, 30, 693-701.

34. Lynch, M.A., Errington, M.L. and Bliss, T.V.P. (1989) The increase in[$^{3}$H]-glutamate release associated with long-term potentiation in the dentate gyrus is blocked by commissural stimulation. Neurosci. Letts., 103, 191-1967.

35. Laroche, R., Redini-Del Negro, C., Clements, M.P. and Lynch, M.A. (1990) Long-term activation of phosphoinositide turnover associated with increased release of amino acids in the dentate gyrus and hippocampus following classical conditioning in the rat. European J. Neurosci, 2, 534-543.

36. Bliss TV, Errington ML, Lynch MA. (1990) Long-term potentiation in the dentate gyrus in vivo is associated with a sustained increase in extracellular glutamate. Adv Exp Med Biol., 268, 269-278.

37. Lynch, M.A. (1989) Mechanisms underlying induction and maintenance of long-term potentiation in the hippocampus. Bioessays, 10, 85-90.

38. Lynch, M.A., Errington, M.L., Clements, M.P., Bliss, T.V.P., Redini Del Negro, C. and Laroche, S. (1989) Increases in glutamate and phosphoinositide metabolism associated with long-term potentiation and classical conditioning. Progr. Brain Res., 83, 251-256.

39. Williams, J.H., Errington, M.L., Lynch, M.A. and Bliss, T.V.P. (1989) Arachidonic acid induces a long-term, activity-dependent enhancement of synaptic transmission in the hippocampus. Nature, 341, 739-742.

40. Lynch, M.A. and Voss, K.L. (1990) Arachidonic acid increases inositol phospholipid metabolism and glutamate release in synaptosomes prepared from hippocampal tissue. J. Neurochem., 55 215-221.

41. Clements, M.P., Errington, M.L., Bliss, T.V.P. and Lynch, M.A. (1989) Time-related changes in basal phosphoinositide turnover after induction of long-term potentiation in the dentate gyrus are blocked by commissural stimulation. Eur. J. Neurosci., 2, 383-387.

42. Bliss, T.V.P., Clements, M.P., Errington, M.L., Lynch, M.A. and Williams, J.H. (1990) Presynaptic changes associated with long-term potentiation in the dentate gyrus. In: Semin. Neurosci., 2.

43. Lynch, M.A. & Voss, K.L. (1991) Presynaptic changes in long-term potentiation: elevated synaptosomal calcium concentration and basal phosphoinositide turnover in dentate gyrus. J. Neurochem, 56, 113-118.

44. Jefferys, J.G.R., Mitchell, P., O'Hara, L., Tiley, C., Hardy, J., Jordan, S.J., Lynch, M.A. and Wadsworth, J. (1991) Ex vivo release of GABA from tetanus toxin induced chronic epileptic foci decreased during the active seizure phase. Neurochem. Int., 18, 373-379.

45. Lynch, M.A., Clements, M.P., Voss, K.L. Bramham, C.R. & Bliss, T.V.P. (1991) Is arachidonic acid a retrograde messenger in long-term potentiation? Biochem. Soc. Trans., 19 391-386.

46. Clements, M.P., Bliss, T.V.P. & Lynch, M.A. (1991) Increase in arachidonic acid concentration in a postsynaptic density fraction following the induction of long-term potentiation in the dentate gyrus. Neuroscience, 45, 379-389.

47. Williams, J.H., Errington, M.L., Li, Y-G., Lynch, M.A. and Bliss, T.V.P. (1993) The search for retrograde messengers in long-term potentiation. Seminars in the Neurosciences, 5, 149-158.,

48. Lynch, M.A., Jaques-Berg, W., Lawson, P.R., Voss, K.L. and Bliss, T.V.P. (1994) The effect of arachidonic acid on uptake in cortical and hippocampal preparations. Neurosci. Res. Comm., 14, 53-61.

49. Lynch, M.A., Voss, K.L., Rodriguez, J. and Bliss, T.V.P. (1994) Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. Neuroscience, 60, 1-5.

50. Lynch, M.A. and Voss, K.L. (1994) Membrane arachidonic acid concentration correlates with age and induction of long-term potentiation in the dentate gyrus. Europ. J. Neurosci., 6, 1008-1014.

51. Lynch, M.A., Voss, K.L. and Gower, A. (1994) Impaired spatial learning in aged rats is associated with alterations in inositol phospholipid metabolism. NeuroReport, 5, 1493-1497.

52. McGahon, B. and Lynch, M.A. (1994) A study of the synergism between metabotropic glutamate receptor activation and arachidonic acid in the rat hippocampus. NeuroReport, 5, 2353-2357.

53. McGahon, B. and Lynch, M.A. (1996) The synergism between metabotropic glutamate receptor activation and arachidonic acid on glutamate release is occluded by induction of long-term potentiation in the dentate gyrus. Neuroscience, 72, 847-855.

54. Cunningham, A.J., Murray, C.A., O'Neill, L.A.J., Lynch, M.A. and O'Connor, J.J. IL-1β inhibits LTP in the rat dentate gyrus *in vitro*: evidence for a role of calcium influx. Neurosci. Letts. (1996) 203, 1-4.

55. McGahon, B. Holscher, C. McGlinchey, L. Rowan, M.J. and Lynch, M.A. Training in the Morris water maze occludes the synergism between ACPD and arachidonic acid on glutamate release in synaptosomes prepared from hippocampus. Learning and Memory (1996) 3, 296-304.

56. McGahon, B. and Lynch, M.A. (1996) The synergism between ACPD and arachidonic acid on glutamate release in hippocampus is age-dependent. Eur. J. Pharmacol., 309, 323-326.

57. Mullany, P., Connolly, S. and Lynch, M.A. (1996) Ageing is associated with changes in glutamate release, protein tyrosine kinase and calcium/calmodulin-dependent protein kinase II in rat hippocampus. Eur. J. Pharmacol., 309, 311-315.

58. Mullany, P.M. and Lynch, M.A. (1997) Changes in protein synthesis and synthesis of the synaptic vesicle protein, synaptophysin, in entorhinal cortex following induction of long-term potentiation in dentate gyrus: an age-related study in the rat. Neuropharmacology, 36, 973-980.

59. Murray, C.A., McGahon, B., McBennett, S. and Lynch, M. (1997) Interleukin-1β inhibits glutamate release in hippocampal synaptosomes prepared from young, but not aged, rats. Neurobiol. Aging, 18, 343-348.

60. McGahon, B., Clements, M.P. and Lynch, M.A. (1997) The ability of aged rats to sustain long-term potentiation is restored when the age-related decrease in membrane arachidonic acid concentration is reversed. Neuroscience, 81, 9-16.

61. McGahon, B., Murray, C.M., Clements, M.P. and Lynch, M.A. (1998) Analysis of the effect of membrane arachidonic acid concentration on modulation of glutamate release by interleukin-1: An age-related study. Exp. Gerontol., 33, 343-354.

62. McGahon, B. and Lynch, M.A. (1998) Analysis of the interaction between arachidonic acid and metabotropic glutamate receptor activation reveals that phospholipase C acts as a coincidence detector in the expression of long-term potentiation in the rat dentate gyrus. Hippocampus, 8, 1-9.

63. Murray, C. and Lynch, M.A. (1998) Evidence that increased hippocampal expression of the cytokine, IL-1β, is a common trigger for age and stress-induced impairments in long-term potentiation. J. Neurosci. 18, 2974-2981.

64. Murray, C. and Lynch, M.A. (1998) Analysis of the mechanism by which dietary supplementation with vitamin E and vitamin C restores ability of aged animals to sustain long-term potentiation in dentate gyrus. J. Biol. Chem. 273, 12161-12168.

65. Campbell, V.A. and Lynch, M.A. (1998) Biphasic modulation of intracellular $Ca^{2+}$ concentration by interleukin-1β in rat cortical synaptosomes: involvement of a PTX-sensitive G-protein and p42 MAP kinase. NeuroReport, 9, 9-12.

66. Kelly, A. and Lynch, M.A. (1998) Evidence that nerve growth factor plays a role in long-term potentiation in the rat dentate gyrus. Neuropharmacology, 37, 561-570.

67. Lynch, M.A. (1998) Age-related impairment in long-term potentiation in hippocampus: A role for the cytokine, interleukin-1β? Progress in Neurobiology, 56, 1-19. Review.

68. Lynch, M.A. (1998) Analysis of the mechanisms underlying the age-related impairment in long-term potentiation in the rat. Reviews in the Neurosciences. 9, 169-201. Review.

69. Mullany, P.M. and Lynch, M.A. (1998) Evidence for a role for synaptophysin in expression of long-term potentiation in rat dentate gyrus. NeuroReport, 9, 2489-2494.

70. O'Donnell, E. and Lynch, M.A. (1998) Age-related changes in handling reactive oxygen species in cortical tissue: effect of dietary manipulation with vitamins E and C. Neurobiol. Ageing, 19, 461-467.

71. Loscher, C.E., Donnelly, S., McBennett, S., Lynch, M.A. and Mills, K.H.G. (1998) Proinflammatory cytokines in the adverse systemic and neurologic effects associated with parenteral injection of a whole cell pertussis vaccine. Ann. N.Y. Acad. Sci., 856, 274-277.

72. Kelly, A. and Lynch, M.A. (1998) LTP occludes the interaction between arachidonic acid and ACPD and NGF and ACPD. NeuroReport, 9, 4087-4091.

73. Campbell, V., Segurado, R. and Lynch, M.A. (1998) Age-related changes in intracellular calcium concentration response to interleukin-1β. Neurobiol. Aging, 19, 575-579.

74. Lynch, M.A. (1999) What is the biological significance of an age-related increase in IL-1β in hippocampus? Mol. Psychiatry, 4, 15-18. Review.

75. McGahon, B., Kelly, A., Maguire, C. and Lynch, M.A. (1999) Activation of p42 mitogen-activated protein kinase by arachidonic acid and ACPD impacts on long-term potentiation in dentate gyrus in the rat: Analysis of age-related changes. Neuroscience, 90, 1167-1175.

76. Murray, C.A., Clements, M.P. and Lynch, M.A. (1999) Interleukin-1 induces lipid peroxidation and membrane changes in rat hippocampus: An age-related study. Gerontology, 45, 136-142.

77. Donnelly, S., Loscher, C., Mills, K.H.G. and Lynch, M.A. (1999) Glycerol-induced seizure: involvement of IL-1β and glutamate. NeuroReport, 10, 1-5.

78. Whittaker, E., Vereker, E. and Lynch, M.A. (1999) Neuropeptide Y inhibits glutamate release and long-term potentiation in rat dentate gyrus. Brain Res. 827, 228-233.

79. McGahon, B.M., Murray, C.A., Horrobin, D.F. and Lynch, M.A. (1999) Age-related changes in

oxidative mechanisms and LTP are reversed by dietary manipulation. Neurobiol. Aging. 20, 643-653.

80. McGahon, B.M., Martin, D.S.D., Horrobin, D.F. and Lynch, M.A. (1999) Age-related changes in synaptic function: Analysis of the effect of dietary supplementation with ω-3 fatty acids. Neuroscience, 94, 305-314.

81. Maguire, C., Casey, M., Kelly, A., Mullany, PM. and Lynch, MA. (1999) Activation of tyrosine receptor kinase, *trk*, plays a role in expression of long-term potentiation in the rat dentate gyrus. Hippocampus, 9, 519-526.

82. Campbell, V., Seguardo, R. and Lynch, M.A. (1999) Regulation of intracellular $Ca^{2+}$ concentration by IL-1β in rat cortical synaptosomes: an age-related study. Neurobiol. Aging, 19, 575-579.

83. McGahon, B.M., Martin, D.SD., Horrobin, D.F. and Lynch, M.A. (1999) Age-related changes in LTP and antioxidant defences are reversed by an α-lipoic acid-enriched diet. Neurobiol. Aging. 20, 655-664

84. Campbell, V and Lynch, M.A. (2000) The role of ceramide ion the modulation of intracellular $Ca^{2+}$ levels by interleukin-1β in rat cortical synaptosomes. Cytokine, 12, 487-490.

85. Loscher, C.E., Donnelly, S. and Lynch, M.A. and Mills, K.H.G. (2000) Induction of inflammatory cytokines in the brain following respiratory infection with *Bordetella Pertussis*. J. Neuroimmunol., 102, 172-181.

86. O'Donnell, E., Vereker, E. and Lynch, M.A. (2000) Age-related impairment in LTP is accompanied by enhanced activity of stress-activated protein kinases: Analysis of underlying mechanisms. Eur. J. Neurosci. 12, 345-352.

87. Kelly, Á., Maguire, C. and Lynch, M.A. (2000) Deficits in NGF release and trk phosphorylation are associated with age-related impairment in LTP in dentate gyrus. Neuroscience, 95, 359-365.

88. Kelly, Á. and Lynch, M.A. (2000) Long-term potentiation in dentate gyrus of the rat is inhibited by the phosphoinositide 3-kinase inhibitor, wortmannin. Neuropharmacology. 39, 643-651.

89. Martin, D.S.D., Towey, M., Horrobin, D.F. and Lynch, M.A. (2000) A diet enriched in α-lipoic acid reverses the age-related compromise in antioxidant defences in rat cortical tissue. Nut Neurosci., 3, 193-206.

90. Vereker, E., O'Donnell E. and Lynch, M.A. (2000) The inhibitory effect of interleukin-1β on LTP is coupled with increased activity of stress-activated protein kinases. J. Neurosci., 20, 6811-6819.

91. Vereker, E., Campbell, V., Roche, E., McEntee, E. and Lynch, M.A. (2000) Lipopolysaccharide inhibits long-term potentiation in the rat dentate gyrus by activating caspase-1. J. Biol Chem., 275, 26252-26528.

92. Loscher, C.E., Donnelly, S., Mills, K.H.G. and Lynch, M.A. (2000) Interleukin-1β-dependent changes in hippocampal function following immunization with a whole cell pertussis vaccine. J. Neuroimmunol., 111, 68-76.

93. Kelly, A., Mullany, P. M. and Lynch, M.A. (2000) Protein synthesis in entorhinal cortex and long-term potentiation in dentate gyrus. Hippocampus, 10, 431-437.

94. Donnelly, S., Loscher, C., Lynch, M.A. and Mills, K.H.G. (2001) Whole cell but not acellular

pertussis vaccines induce convulsive activity in mice: evidence of a role for toxin-induced IL-1β in a new murine model for analysis of neuronal side effects of vaccination. Infect Immun., 69, 4217-4223.

95. Gooney, M. and Lynch, M.A. (2001) Long-term potentiation in the dentate gyrus of the rat hippocampus is accompanied by brain-derived neurotrophic factor-induced activation of TrkB. J. Neurochem., 77, 1198-207.

96. Lynch, M.A. (2001) Lipoic acid confers protection against oxidative injury in non-neuronal and neuronal tissue. Nut. Neurosci. 4, 419-438.

97. Vereker, E., O'Donnell, E., Lynch, A.M., Kelly, Á., Nolan, Y. and Lynch, M.A. (2001) Evidence that interleukin-1β and reactive oxygen species production play a pivotal role in stress-induced impairment of long-term potentiation in the rat dentate gyrus. Eur. J. Neurosci. 14, 1809-1819.

98. Kelly, A., Lynch, A., Vereker, E., Nolan, Y., Queenan, P., Whittaker, E., O'Neill, L.A. and Lynch, M.A. (2001) The anti-inflammatory cytokine, IL-10, blocks the inhibitory effect of IL-1beta on LTP: A role for JNK. J. Biol. Chem., 276, 45564-45572.

99. Lynch, M.A. (2002) IL-1β exerts a myriad of effects in the brain and in particular in the hippocampus: Analysis of some of these actions. Vitamins and Hormones, 64, 185-219. Review.

100. Gooney, M.A., Shaw, K., Kelly, Á., O'Mara, S.M. and Lynch, M.A. (2002) Long-term potentiation and spatial learning are associated with increased phosphorylation of *TrkB* and extracellular signal regulated kinase (ERK) in dentate gyrus: Evidence for a role for brain-derived neurotrophic factor. Behav. Neurosci. 116, 455-463.

101. Casey, M., Maguire, C., Kelly, Á., Gooney, M.A. and Lynch, M.A. (2002) Analysis of the presynaptic signalling mechanisms underlying the inhibition of LTP in rat dentate gyrus by the tyrosine kinase inhibitor, genistein. Hippocampus, 12, 377-385.

102. Lynch, M.A. (2002) IL-1β exerts a myriad of effects in the brain and in particular in the hippocampus: Analysis of some of these actions. Vitamins and Hormones, 64, 185-219. Review.

103. Lonergan, P.E., Martin, D.S.D., Horrobin, D.F. and Lynch, M.A. (2002) Neuroprotective effect of eicosapentanoate in hippocampus of rats exposed to Gamma irradiation. J. Biol. Chem., 277, 20804-20811.

104. Lynch, A.M. and Lynch, M.A. (2002) The age-related increase in IL-1 Type I receptor in rat hippocampus is coupled with an increase in caspase-3 activation. Eur. J Neurosci., 15, 1779-1788.

105. Nolan, Y., Minogue, A., Vereker, E., Bolton, A.E., Campbell, V.A. and Lynch, M.A. (2002) Attenuation of LPS-induced changes in synaptic activity in rat hippocampus by Vasogen's Immune Modulation Therapy. Immunomodulation, 10, 40-46.

106. Martin, D.S.D, Spencer, P., Horrobin, D.F. and Lynch, M.A. (2002) Long-term potentiation in aged rats is restored when the age-related decrease in polyunsaturated fatty acid concentration is reversed. Prostaglandins, Leukotrienes and Essential Fatty Acids, 67, 121-130

107. Martin, D.S., Lonergan, P.E., Boland, B., Fogarty, M.P., Brady, M., Horrobin, D.F., Campbell, V.A. and Lynch, M.A. (2002) Apoptotic changes in the aged brain are triggered by interleukin-1beta -induced activation of p38 and reversed by treatment with eicosapentaenoic acid. J. Biol. Chem., 277, 34239-34246.

108. Hauss-Wegrzyniak, B., Lynch, M.A., Vraniak, P.D. and Wenk, G.L. (2002) Chronic brain inflammation results in cell loss in the entorhinal cortex and impaired LTP in perforant path-granule cell synapses. Exp. Neurol., 176, 336-341.

109. Kelly, A., Vereker, E., Brady, M., Barry, C., Loscher, C., Mills, K.H.G. and Lynch, M.A. (2003) Activation of p38 plays a pivotal role in the inhibitory effect of lipopolysaccharide and interleukin-1β-induced inhibition of long-term potentiation in rat dentate gyrus. J. Biol. Chem. 278, 19453-19462

110. Loscher CE, Mills KH, Lynch MA. (2003) Interleukin-1 receptor antagonist exerts agonist activity in the hippocampus independent of the interleukin-1 type I receptor. J Neuroimmunol. 137, 117-124.

111. Armstrong ME, Loscher CE, Lynch MA, Mills KH. IL-1beta-dependent neurological effects of the whole cell pertussis vaccine: a role for IL-1-associated signalling components in vaccine reactogenicity. J Neuroimmunol. 136, 25-33.

112. Dhanrajan, T.M., Lynch, M.A., Kelly, A., Popov, V.I., Rusakov, D.A. and Stewart, M.G. (2003) Expression of long term potentiation in aged rats involves perforated synapses but dendritic spine branching results from high frequency stimulation alone. Hippocampus. In the press

113. Minogue, A.M., Schmid, A.W., Fogarty, M.F., Moore, A.C., Campbell, V.A. and Herron, C.E. (2003) Activation of the JNK signalling cascade mediates the effect of Aβ on LTP and cell death in hippocampus: A role for IL-1β. J. Biol. Chem., 278, 27971-27980.

114. Lynch, M.A. (2004) LTP and memory. Physiol. Rev., 84, 87-136.

115. Davies HA, Kelly A, Dhanrajan TM, Lynch MA, Rodri;guez JJ, Stewart MG. (2003) Synaptophysin immunogold labelling of synapses decreases in dentate gyrus of the hippocampus of aged rats. Brain Res., 986, 191-195.

116. Lynch AM, Moore M, Craig S, Lonergan PE, Martin DS, Lynch MA. (2003) Analysis of IL-1beta -induced cell signaling activation in rat hippocampus following exposure to gamma irradiation: protective effect of eicosapentaenoic acid. J Biol Chem. 51, 51075-51084

117. Nolan, Y., Vereker, E., Lynch, AM and Lynch, MA (2003) Evidence that lipopolysaccharide-induced cell death is mediated by accumulation of reactive oxygen species and activation of p38 in rat cortex and hippocampus. Exp Neurol. 184, 794-804.

118. FO Maher, DSD Martin and MA Lynch (2004) Increased IL-1β in cortex of aged rats is accompanied by downregulation of ERK and PI-3 kinase. Neurobiol. Aging, 25, 795-806

119. Lynch AM, Walsh C, Delaney A, Nolan Y, Campbell VA, Lynch MA. (2004) Lipopolysaccharide-induced increase in signalling in hippocampus is abrogated by IL-10 - a role for IL-1beta? J. Neurochem., 88, 635-646

120. Nolan, Y., Martin D.S.D., Campbell, V.A. and Lynch, M.A. (2004) Evidence of a protective effect of phosphatidylserine on lipopolysaccharide-induced impairment of long-term potentiation in the rat hippocampus. J. Neuroimmunol., 151, 12-23.

121. Gooney, M., Messaoudi, E., Maher, F.O., Bramham, C.R. and Lynch, M.A. (2004) BDNF-induced LTP is associated with enhanced release of endogenous BDNF and glutamate and presynaptic activation of CREB: analysis of deficits in aged rats. Neurobiol. Aging In the press.

122. Kavanagh, T., Lonergan, P.E. and Lynch, M.A. (2004) Eicosapentaenoic acid and gamma-linolenic acid increase hippocampal concentrations of IL-4 and IL-10 and abrogate lipopolysaccharide-induced inhibition of long-term potentiation. Prostaglandins Leukot Essent Fatty Acids, 70, 391-397.

Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2002–03;10:40–46

Received: February 28, 2002
Accepted: April 3, 2002



# Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy

Yvonne Nolan[a] Aedín Minogue[a] Emily Vereker[a] Anthony E. Bolton[b]
Veronica A. Campbell[a] Marina A. Lynch[a]

[a]Department of Physiology, Trinity College, Dublin, Ireland; [b]Vasogen Inc., Toronto, Canada

**Key Words**

Long-term potentiation · Hippocampus · IL-10 · IL-1β · Lipopolysaccharide · Vasogen's IMT

**Abstract**

Systemic injection of lipopolysaccharide (LPS) blocks the expression of long-term potentiation in the hippocampus of the rat. This is coupled with increased IL-1β concentration and c-Jun $NH_2$-terminal kinase activity, as well as an increase in the number of cells displaying apoptotic characteristics in the hippocampus. Vasogen's Immune Modulation Therapy (IMT) is a procedure involving intramuscular administration of syngeneic blood which has been exposed ex vivo to elevated temperature, oxidation and ultraviolet light. We report that Vasogen's IMT significantly abrogates these LPS-induced effects with a concomitant increase in the concentration of the anti-inflammatory cytokine IL-10. These data suggest that Vasogen's IMT may play a protective role against the deleterious effects of immune insults in the brain.



## Introduction

Systemic administration of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, provokes activation of the immune system by inducing an increase in pro-inflammatory cytokines such as IL-1β. As well as its effects on the peripheral immune system, LPS is now known to be responsible for stimulating changes in the central nervous system, affecting processes such as thermoregulation, sleep and appetite [1]. One example of a neuronal deficit induced by LPS and IL-1β is the impairment of long-term potentiation (LTP) in the hippocampus [2, 3]. LTP is a form of synaptic plasticity and has been proposed as a biological substrate for learning and memory [4]. The inhibitory effects of both IL-1β and LPS on LTP have been linked with an increase in activity of the stress-activated protein kinase c-Jun $NH_2$-terminal kinase (JNK) [2]. Activation of JNK has been identified as instrumental in bringing about cell function deterioration and, ultimately, cell death [5, 6].

IL-10 is one of a number of cytokines secreted by the T helper 2 (Th2) subclass of lymphocytes and is known for its anti-inflammatory effects. Anti-inflammatory cytokines, such as IL-10, have been reported to prevent IL-1β-induced changes [7, 8], thus inhibiting pro-inflammatory responses. It has been demonstrated that Vasogen's Immune Modulation Therapy (IMT), which involves intramuscular administration of syngeneic blood following ex vivo treatment with elevated temperature, oxidation

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com


1021–7401/02/0101–0040$18.50/0

Accessible online at:
www.karger.com/journals/nim

Dr. Marina A. Lynch
Department of Physiology
Trinity College
IRL–Dublin 2 (Ireland)
Tel. +353 1 608 1770, Fax +353 1 679 3545, E-Mail lynchma@tcd.ie

and ultraviolet light [9], appears to mediate a Th1 to Th2 immunodeviation in patients suffering from scleroderma, a Th1-mediated disease [10]. There is evidence that Vasogen's IMT suppresses contact hypersensitivity [11] and reduces the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice [12], suggesting a possible anti-inflammatory activity. The proposed role of Vasogen's IMT in interfering with pro-inflammatory responses prompted an investigation into its effect on LPS-induced neuroinflammation. In this study we assessed changes in LTP, IL-1β concentration, JNK activity and evaluated TUNEL staining for fragmented DNA, a characteristic of apoptotic cells.

## Materials and Methods

*Animals and Treatment Protocol*

Male Wistar rats (300–350 g; BioResources Unit, Trinity College Dublin, Ireland) were used in these experiments. Animals were housed in groups of 4 under a 12-hour light/dark schedule with free access to food and water. Ambient temperature was controlled between 22 and 23°C and rats were maintained under veterinary supervision.

Whole blood (11 ml) was obtained from donor rats by cardiac puncture and added to 2.2 ml of 3.13% sodium citrate solution, of which 1.2 ml was removed and used for sham treatment. For Vasogen's IMT, the remaining 12 ml of anticoagulated blood were transferred to a single-use blood container (VC7002, Vasogen Inc, Toronto, Canada) and exposed to a combination of controlled physiochemical stress factors in a medical device (VC7001, Vasogen Inc.). The medical device executed an automated procedure during which the temperature of the blood was first raised (to a nominal temperature of 42.5°C) over a period of 6–8 min. A gas mixture of ozone in medical oxygen (nominal concentration 14.5 μg/ml) was then applied to the blood (nominal flow rate 240 ml/min) for 3 min. During this time the blood was exposed to UVC light (maximum emission spectrum at 254 nm). Finally, the treated blood was allowed to settle for at least 7 min prior to removal from the blood container. Rats were treated by intramuscular injection of 150 μl of processed blood or untreated blood (sham treatment). Injections were administered 14 days, 13 days and 1 day before LPS or saline challenge. Rats were divided into 4 treatment groups, which will be referred to as sham-saline, sham-LPS, IMT-saline, IMT-LPS.

*Induction of LTP in Perforant Path-Granule Cell Synapses in vivo*

LTP was induced as described previously [13]. Rats were anaesthetised by an intraperitoneal urethane injection (1.5 g/kg), subsequently received either LPS (100 μg/kg) or saline intraperitoneally and were monitored for 3 h. Rats were then placed in a head holder in a stereotaxic frame. A window of skull was removed to allow placement of recording and stimulating electrodes in the molecular layer of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma) and perforant path (angular bundle, 4.4 mm lateral to lambda), respectively. The depth of the electrodes was adjusted to obtain maximal responses in the cell body region and, after an initial period to allow baseline responses to stabilise, test shocks were delivered to the perforant path at the rate of 1/30 s. Responses were recorded for 10 min prior to and 40 min following tetanic stimulation (3 trains of stimuli; 250 Hz for 200 ms; intertrain interval 30 s). At the end of the electrophysiological recording period, rats were killed by decapitation, the hippocampus was removed, dissected on ice and cross-chopped into slices (350 μm × 350 μm), using a McIlwain tissue chopper. The time needed to prepare slices from the time of death was 2.5–3.5 min. All samples were frozen separately in 1 ml Krebs solution (composition, in m*M*: NaCl 136, KCl 2.54, $KH_2PO_4$ 1.18, $MgSO_4 \cdot 7\ H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) containing 10% dimethylsulphoxide [14]. For analysis, thawed slices of tissue were rinsed 3 times in fresh ice-cold Krebs solution and homogenized in ice-cold Krebs solution.

*Analysis of IL-1β Concentration*

IL-1β concentration in hippocampal homogenate was analysed by ELISA (R & D Systems, UK). Antibody-coated (100 μl; 1.0 μg/ml final concentration, diluted in phosphate buffered saline (PBS), pH 7.3; goat anti-rat IL-1β antibody), 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with 300 μl blocking buffer (PBS, pH 7.3, containing 5% sucrose, 1% bovine serum albumin (BSA), and 0.05% $NaN_3$). After several washes, plates were incubated with IL-1β standards (100 μl; 0–1,000 pg/ml in PBS containing 1% BSA) or samples (homogenised in Krebs solution containing 2 m*M* $CaCl_2$) for 2 h at room temperature. Samples were incubated with secondary antibody (100 μl; final concentration 350 ng/ml in PBS containing 1% BSA and 2% normal goat serum; biotinylated goat anti-rat IL-1β antibody) for 2 h at room temperature, washed and incubated in detection agent (100 μl; horseradish peroxidase conjugated streptavidin; 1:200 dilution in PBS containing 1% BSA) for 20 min at room temperature. Substrate solution (100 μl; 1:1 mixture of $H_2O_2$ and tetramethylbenzidine) was added, samples were incubated at room temperature in the dark for 1 h, the reaction was stopped using 50 μl 1M $H_2SO_4$. Absorbance was read at 450 nm, values were corrected for protein [15] and expressed as pg/mg protein.

*Analysis of IL-10 Concentration*

A commercially available ELISA (Biosource International Inc., USA) was used to analyse IL-10 concentration in the hippocampus. Samples were homogenised in Iscove's culture medium containing 5% fetal bovine serum and a cocktail of enzyme inhibitors (100 m*M* amino-n-caproic acid; 10 m*M* $Na_2EDTA$; 5 m*M* benzamidine HCl; 0.2 m*M* phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min, the supernatant was removed and analysed for IL-10 using ELISA. Optical densities were determined at 450/630 nm dual wavelength mode using a multi-well plate reader; values were corrected for protein [15] and expressed as pg/mg protein.

*Analysis of JNK Activity*

The activity of JNK was analysed in homogenate prepared from frozen hippocampal slices. In a separate experiment, activity of the kinase was assessed in freshly prepared hippocampal synaptosomes obtained from untreated rats. These samples had been pre-treated for 20 min in the absence and presence of IL-1β (1 ng/ml) and vasoactive intestinal peptide (VIP; 1 μM), a proven JNK inhibitor [16]. In all experiments, samples were analysed for protein [15], and diluted to

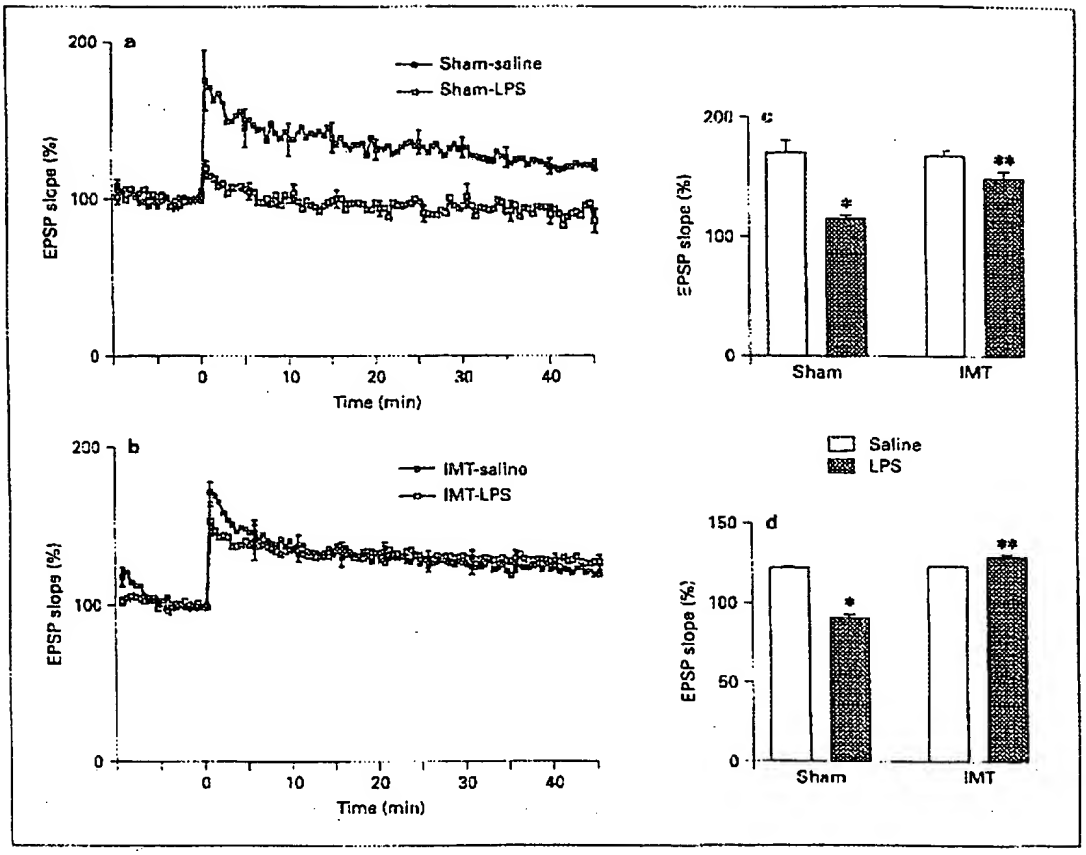



**Fig. 1.** Intraperitoneal injection of LPS inhibits LTP in perforant path-granule cell synapses (**a**). This inhibitory effect is prevented by pre-treatment with Vasogen's IMT, which exerted no significant effect in saline-treated rats (**b**). The data presented are means of 7–8 observations in each treatment group. Data are expressed as mean percentage change in EPSP slope every 30 s, normalised with respect to the mean value in the 5 min immediately prior to tetanic stimulation. SEM are included for every 10th response. Analysis of the mean values in the 2 min immediately following tetanic stimulation (**c**) and in the last 5 min of the experiment (**d**) indicate that population EPSP slope was significantly decreased in the sham-LPS group (* $p < 0.01$ vs. sham-saline; ANOVA), but that Vasogen's IMT significantly reversed this effect (** $p < 0.01$ vs. sham-LPS; ANOVA). These values are means ± SEM of 7–8 observations in each case.

equalise for protein concentration. These samples (10 µl, 1 mg/ml) were added to 10 µl sample buffer (Tris-HCl 0.5 m*M*, pH 6.8; glycerol 10%; SDS 10%; β-mercaptoethanol 5%; bromophenol blue 0.05% w/v), boiled for 5 min and loaded onto gels (10% SDS). Proteins were separated by application of a 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min) and immunoblotted by incubation with an antibody that specifically targets phosphorylated JNK [Santa Cruz Biotechnology, USA; 1:200 in Tris-buffered saline Tween (0.1% Tween-20) containing 1% BSA] for 2 h at room temperature. Nitrocellulose strips were washed and incubated for 2 h at room temperature with secondary antibody (peroxidase-linked anti-mouse IgG; 1:300 dilution; Sigma, UK). Visualization was achieved using SuperSignal West Dura Extended Duration Substrate (Pierce, USA). Immunoblots were immersed in substrate for 5 min and subsequently exposed to film for 1 s. Film was processed using a Fuji X-ray processor, and quantification of protein bands was achieved by densitometric analysis using two software packages: Grab It (Grab It Annotating Grabber 2.04.7, Synotics; UVP Ltd., UK) and Gelworks (Gelworks ID, Version 2.51; UVP Ltd) for photography and densitometry, respectively. Gelworks provides a single value (in arbitrary units), representing the density of each blot; the values presented here are means of data generated from at least 4 separate experiments.

*TUNEL Staining*

Dissociated cells were prepared by enzymatic and mechanical digestion of fresh hippocampal slices. Slices were incubated with collagenase (0.125%; Sigma) in PBS for 30 min at room temperature,

washed with PBS to terminate collagenase digestion, and then gently triturated with a glass Pasteur pipette before passing through a nylon mesh filter to remove tissue clumps. Cells were then cytospun onto glass microscope slides, fixed with methanol and stored until use.

TUNEL (Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labelling) staining, which identifies nuclei with fragmented DNA (a characteristic of apoptotic cells), was performed according to the manufacturer's (Promega, USA) instructions. Briefly, fixed cytospun cells were washed and permeabilised with 0.2% Triton in PBS. Cells were equilibrated in buffer (200 m*M* potassium cacodylate (pH 6.6 at 25°C), 25 m*M* Tris-HCl (pH 6.6 at 25°C), 0.2 m*M* DTT, 0.25 mg/ml BSA, 2.5 m*M* $CoCl_2$) for 5 min at room temperature and incubated in TdT reaction mixture (30 µl; 98 µl equilibration buffer, 1 µl biotinylated nucleotide mix, 1 µl TdT enzyme) at 37°C for 1h. The reaction was terminated by adding 100 µl 2 × SCC (1:10; 2 × SCC:deionised water), endogenous peroxidases were blocked by incubation with $H_2O_2$ (100 µl; 0.3% in PBS) for 5 min at room temperature, and washed cells were incubated for 30 min at room temperature in streptavidin HRP solution (100 µl; 1:500 in PBS) to allow binding to the biotinylated nucleotides. Diaminobenzidine solution was added to washed cells, and the incubation proceeded for 10 min at room temperature. Cells were washed with deionised water, dehydrated through graded ethanol, cleared with xylene and then slides were mounted in DPX mounting medium and coverslipped. TUNEL-positive cells were expressed as a percentage of the total.

*Statistical Analysis*

Data were analysed, as appropriate, using either Student's t test for independent means, or a one-way analysis of variance (ANOVA) followed by post hoc Student Newman-Keuls test to determine which conditions were significantly different from each other. Data are expressed as means with standard errors and deemed statistically significant when $p < 0.05$.

## Results

In the sham-LPS group, tetanic stimulation delivered to the perforant path 3 h after intraperitoneal injection of LPS resulted in an increase in the mean slope of the population excitatory post-synaptic potential (EPSP) recorded in cell bodies of the granule cells. The mean percentage change (± SEM) in the 2 min immediately following tetanic stimulation compared with 5 min immediately before tetanic stimulation was 114.49 ± 2.79. This was not maintained, however, so that the mean percentage change in population EPSP slope in the last 5 min of the experiment was 90.32 ± 2.42 in the sham-LPS group. The corresponding values in the sham-saline groups of rats were 170.15 ± 10.16 and 121.28 ± 1.20, respectively (fig. 1a).

The LPS-induced inhibition of LTP was overcome by pre-treatment with Vasogen's IMT. The mean percentage change in population EPSP slope (mean ± SEM) in the 2 min immediately after tetanic stimulation was 147.44 ± 5.84 in the IMT-LPS group compared with 166.85 ± 4.54 in the IMT-saline group. In the last 5 min of the experiment, the values were 128.07 ± 1.46 for the IMT-LPS group and 121.96 ± 0.85 for the IMT-saline group ($n = 7–8$; fig. 1b). The values in the 2 min immediately after tetanic stimulation and in the last 5 min of the experiment were similar in sham-saline and IMT-saline groups ($p > 0.05$). Statistical analysis of the data (fig. 1c, d) revealed that both the early and late phases of LTP were markedly reduced by LPS administration in sham-treated rats ($p < 0.01$ in both cases; ANOVA), and that pre-treatment with Vasogen's IMT significantly attenuated the effect of LPS ($p < 0.01$ in both cases; ANOVA).

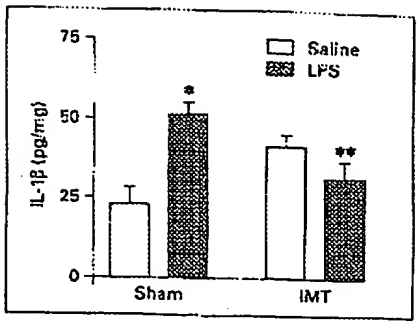



**Fig. 2.** LPS stimulated a significant increase in the concentration of hippocampal IL-1β in sham-treated animals (* $p < 0.01$ vs. sham-saline; ANOVA). This effect was significantly abrogated in animals pre-treated with Vasogen's IMT (** $p < 0.05$ vs. sham-LPS; ANOVA). Data are expressed as means ± SEM, $n = 7–8$.

Figure 2 shows that IL-1β concentration in the hippocampus was significantly increased in the sham-LPS group compared to the sham-saline group ($p < 0.01$; ANOVA); this increase was significantly attenuated by pre-treatment with Vasogen's IMT ($p < 0.05$; ANOVA).

The LPS-induced changes in IL-1β and LTP in sham-LPS rats were associated with an increase in JNK activity in the hippocampus (fig. 3a). In animals treated with Vasogen's IMT, however, these differences were coupled with an attenuated LPS-induced increase in JNK ($p < 0.05$; ANOVA; fig. 3a). Thus JNK activation was increased in the hippocampi of sham-treated animals challenged with LPS [compare lanes 1 (sham-saline) and 2 (sham-LPS)]. This effect was attenuated in rats treated with Vasogen's IMT [compare lanes 2 (sham-LPS) and 4 (IMT-LPS)], which exerted no effect if given on its own [lane 3 (IMT-saline)]. Mean data obtained from densitometric analysis revealed that LPS challenge significantly increased JNK activation in sham-treated animals by

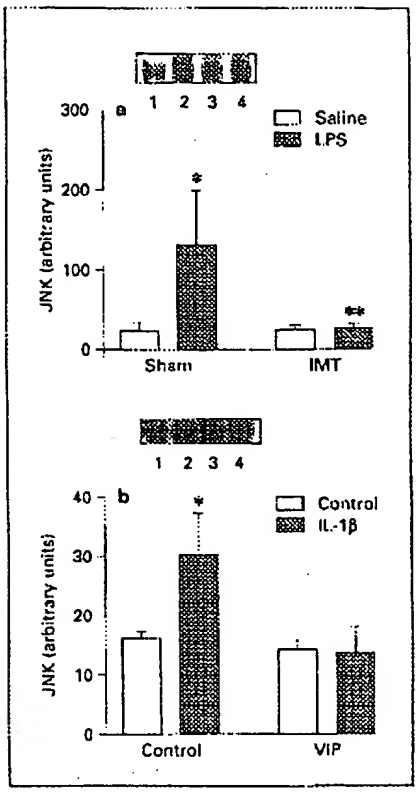



**Fig. 3.** The LPS-induced increase in JNK activity is blocked by pre-treatment with Vasogen's IMT (**a**). LPS induces a significant increase in JNK activity in the hippocampi of sham-treated rats, as indicated by an increase in the phosphorylated form of JNK (JNK-1 isoform 46 kD; * $p < 0.05$ vs. sham-saline; ANOVA). Analysis of the mean data obtained from densitometric analysis indicated that Vasogen's IMT significantly reduced this effect of LPS. (** $p < 0.05$ vs. sham-LPS; ANOVA). Sample immunoblots indicate the stimulatory effects of LPS (lane 2) on JNK activity in the absence of Vasogen's IMT (compare lanes 1 and 2) and the inhibition of this effect after pre-treatment with Vasogen's IMT (compare lanes 2 and 4). Data are expressed as means ± SEM, n = 7–8. In vitro, VIP blocks IL-1β-induced increase in JNK activity (**b**). IL-1β induces a significant increase in JNK activity (* $p < 0.05$ vs. control; ANOVA; compare lanes 1 and 2), but this effect is blocked by co-incubation with VIP (compare lanes 2 and 4). Data are expressed as means ± SEM, n = 6.

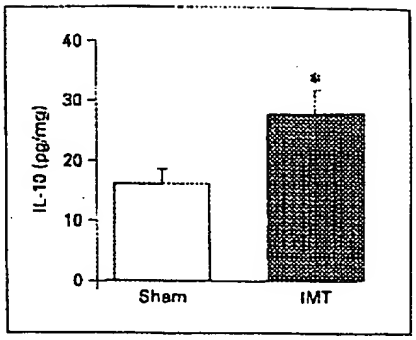



**Fig. 4.** The concentration of anti-inflammatory cytokine IL-10 was significantly increased in hippocampal tissue as a result of treatment with Vasogen's IMT (* $p < 0.05$ vs. sham; Student's t test for independent means). Data are expressed as means ± SEM, n = 7–8.

LPS challenge ($p < 0.05$; ANOVA), whereas no parallel effect of LPS on JNK activation was observed in tissue prepared from rats treated with Vasogen's IMT.

When JNK activity was analysed after pre-incubating freshly prepared hippocampal synaptosomes from untreated rats in the absence and presence of IL-1β and the non-specific JNK inhibitor VIP, a VIP-associated attenuation of IL-1β-induced activity was observed (fig. 3b). In vitro, IL-1β induced a significant increase in JNK activity ($p < 0.05$; ANOVA; compare lanes 1 and 2), but this effect was blocked by co-incubation with VIP (compare lanes 2 and 4).

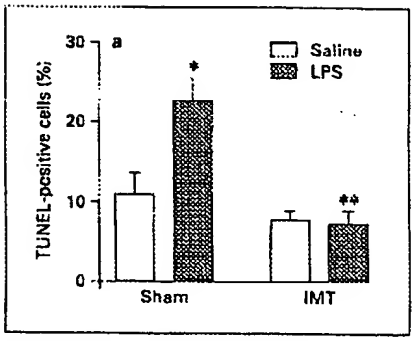
Analysis of IL-10 in the rat hippocampus revealed that Vasogen's IMT was associated with a significant increase in IL-10 relative to sham treatment ($p < 0.05$; Student's t test for independent means; fig. 4).

Figure 5a demonstrates that the percentage of dissociated cells prepared from fresh hippocampal tissue staining positive for TUNEL was significantly increased in the sham-LPS group compared with the sham-saline group ($p < 0.01$; ANOVA). Animals treated with Vasogen's IMT did not display this degenerative effect of LPS ($p < 0.01$; ANOVA). A representative image of TUNEL-positive cells shows an increased number of apoptotic cells after LPS injection, as evidenced by increased number of cells displaying dark brown stained nuclei, i.e. TUNEL-positive cells (fig. 5bii). This contrasts with cells prepared from hippocampi of sham-saline (fig. 5bi) and IMT-saline rats (fig. 5biii). Figure 5biv shows a reduction in the number of cells displaying TUNEL-positive staining in the IMT-LPS group.

## Discussion

The objective of this study was to investigate the possibility that pre-treating rats with Vasogen's IMT may block the LPS-induced inhibitory effects on synaptic plasticity in the hippocampus. Accordingly, the data demonstrate that the LPS-induced inhibition of LTP in perforant path-granule cell synapses was abrogated by pre-treatment with Vasogen's IMT.

Systemic injection of LPS in sham-treated animals induced an increase of IL-1β in the hippocampus, a find-



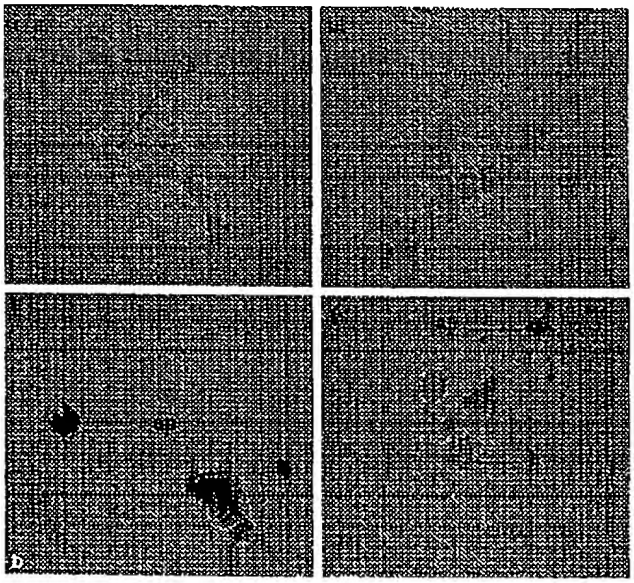




**Fig. 5.** The LPS-induced apoptotic changes in hippocampal cells were inhibited by pre-treatment with Vasogen's IMT. Cytospun cells were prepared from the hippocampi of rats and the mean data, obtained by counting 200 cells on each coverslip, show a significant increase in the percentage of apoptotic cells in the sham-LPS group compared with sham-saline rats (* $p < 0.01$ vs. sham-saline; ANOVA). This percentage increase is reversed by pre-treatment with Vasogen's IMT (** $p < 0.01$ vs. sham-LPS; ANOVA; **a**). Data are expressed as mean ± SEM. $n = 5$. Representative image TUNEL staining displays healthy (h) and apoptotic (ap) cells (**b**). There is an increased number of dark brown stained cells prepared from hippocampi of rats injected with LPS (ii) compared with cells prepared from saline-injected control rats (i), and rats treated with IMT only (iii). Pre-treatment with IMT reversed the effects of LPS with fewer cells displaying brown staining (iv). Scale bar is 20 μm.

ing that supports earlier reports [2, 17]. It has been proposed that LPS may inhibit LTP in perforant path-granule cell synapses as a consequence of an LPS-induced increase in IL-1β concentration in the hippocampus [2]. Indeed, it has previously been reported that intracerebroventricular injection of IL-1β inhibits LTP in perforant path-granule cell synapses in vivo [3, 8], and that IL-1β attenuates LTP in dentate gyrus in vitro [18]. Pre-treatment with Vasogen's IMT prevented the LPS-stimulated increase in hippocampal IL-1β concentration, as well as the LPS-induced inhibition of LTP.

Data from this laboratory have demonstrated a stimulatory effect of both LPS and IL-1β on JNK activation [2, 19]. As a consequence of the preventative effect of Vasogen's IMT on the LPS-induced increase in IL-1β concentration, the stimulatory effect of LPS on JNK activity was also attenuated. Here we also report that co-incubation of synaptosomes in the presence of IL-1β and the non-specific JNK inhibitor VIP blocks the IL-1β-induced increased activation of JNK. Thus it seems reasonable to propose that Vasogen's IMT may exert its protective effect on synaptic function by acting to prevent this LPS-induced signalling event.

Another consequence of peripheral administration of LPS is neuronal degeneration, as demonstrated by increased numbers of cells whose nuclei display fragmented DNA, a characteristic associated with apoptosis [2, 6, 20]. Accordingly, we have demonstrated a significant increase in the percentage of TUNEL-positive cells in the hippocampus as a result of LPS administration in sham-treated animals. It is possible that the impairment in LTP due to LPS injection in sham-treated animals may be due to degenerative changes in hippocampal cells. Thus it is not unreasonable to suggest that the prevention of LPS-induced inhibition of LTP by Vasogen's IMT is paralleled by a prevention of LPS-induced cell death. The present evidence, which shows an abrogation of LPS-induced increase in TUNEL-positive hippocampal cells by Vasogen's IMT, supports this proposal. Concurrent with this finding is the observation that pre-treatment with Vasogen's IMT reduces apoptosis after acute renal ischemia/reperfusion injury in dogs, as estimated by the reduction in mitochondrial membrane potential [21].

It has recently been shown that intracerebroventricular administration of the anti-inflammatory cytokine IL-10 in vivo reverses IL-1β-induced impairment of LTP and JNK activation in the hippocampus [8]. Injection of IL-10 has also been shown to reduce LPS-induced fever [22] and the behavioural effects induced by LPS [23]. In addition, IL-10 has been shown to confer protection against oligo-

dendroglial death evoked by LPS/IFN-γ in vitro [24]. In the present study, pre-treatment with Vasogen's IMT caused a significant increase in IL-10 concentration in the hippocampus. This finding is consistent with the observation that administration of Vasogen's IMT inhibits Th1-mediated contact hypersensitivity in mice [11], and the level of suppression was comparable to that seen with animal models using IL-10 [25]. Therefore, it is possible that in the present experimental paradigm, Vasogen's IMT may exert its beneficial effects by suppressing the Th1 response with a concomitant release of anti-inflammatory IL-10, as suggested by the obstruction of LPS-induced pro-inflammatory effects. Whether the IMT-induced central decrease in IL-1β and the increase in IL-10 observed in the study are due to a peripheral decrease in IL-1β and increase in IL-10 is as yet unknown. Current information on the mechanism of immune molecule trafficking across the blood-brain barrier is limited. However, some signals that regulate immune cell traffic include intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on activated brain endothelia, and their counter-receptors LFA-1 and VLA-4 on immune cells. It remains to be investigated whether peripheral administration of Vasogen's IMT induces its central effects by activation of these mediators.

Although the exact mechanism of action of Vasogen's IMT remains to be elucidated, there is clear evidence from the data presented in this study that pre-treatment with the therapy confers a protective effect on the organism by preventing LPS-induced impairment of synaptic function and the resultant detrimental effects in the hippocampus of the rat.

## References


1 Linthorst ACE, Reul JMH: Brain neurotransmission during peripheral inflammation. Ann NY Acad Sci 1998;840:139–152.

2 Vereker E, Campbell V, Roche E, McEntee E, Lynch MA: Lipopolysaccharide inhibits long term potentiation in the rat dentate gyrus by activating caspase-1. J Biol Chem 2000;275: 26252–26258.

3 Murray CA, Lynch MA: Evidence that increased hippocampal expression of the cytokine interleukin-1β is a common trigger for age- and stress-induced impairments in long-term potentiation. J Neurosci 1998;18:2974–2981.

4 Bliss TV, Collingridge GL: A synaptic model of memory: Long-term potentiation in the hippocampus. Nature 1993;361:31–39.

5 Maroney AC, Glicksman MA, Basma AN, Walton KM, Knight E, Murphy CA, Bartlett BA, Finn JP, Angeles T, Matsuda Y, Neff NT, Dionne CA: Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. J Neurosci 1998: 18:104–111.

6 Campbell V, Roche E, Lynch MA: Intraperitoneal administration of lipopolysaccharide induces apoptosis in rat entorhinal cortex. J Physiol (Lond) 2000;523:189.

7 Oriss TB, McCarthy SA, Morel BF, Campana MA, Morel PA: Crossregulation between T helper cell (Th) 1 and Th2: Inhibition of Th2 proliferation by IFN-γ involves interference with IL-1. J Immunol 1997;158:3666–3672.

8 Kelly A, Lynch A, Vereker E, Nolan Y, Queenan P, Whittaker E, O'Neill LAJ, Lynch MA: The anti-inflammatory cytokine, interleukin (IL)-10, blocks the inhibitory effect of IL-1β on LTP. A role for JNK. J Biol Chem 2001;276: 45564–45572.

9 Bulmer J, Bolton AE, Pockley AG: Effect of combined heat, ozonation and ultraviolet irradiation (VasoCare) on heat shock protein expression by peripheral blood leukocyte populations. J Biol Regul Homeost Agents 1997;11: 104–110.

10 Rabinovich BA, Matukas L, Raju K, Punhani T, deVeber G, Keystone E: VasoCare™ PSCT normalizes the Th2 cell subset in scleroderma. Proceedings of the XII Pan-American Congress of Rheumatology, Montreal, 1998.

11 Shivji GM, Suzuki H, Mandel AS, Bolton AE, Sauder DN: The effect of VAS972 on allergic contact hypersensitivity. J Cutan Med Surg 2000;4:132–137.

12 Babaci S, Stewart DJ, Picard P, Monge JC: Effects of VasoCare therapy on the initiation and progression of atherosclerosis. Atherosclerosis 2002;162:45–53.

13 McGahon B, Lynch MA: The synergism between metabotropic glutamate receptor activation and arachidonic acid on glutamate release is occluded by induction of long-term potentiation in the dentate gyrus. Neuroscience 1996; 72:847–855.

14 Haen EA, Bowen DM: Protection of neocortical tissue prisms from freeze-thaw injury by dimethyl sulphoxide. J Neurochem 1981;37: 243–246.

15 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.

16 Delgado M, Ganea D: Vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide inhibit the MEKK1/MEK4/JNK signalling pathway in LPS-stimulated macrophages. J Neuroimmunol 2000;110:97–105.

17 Hansen MK, Nguyen KT, Gochler LE, Gaykema RP, Fleshner M, Maier SF, Watkins LR: Effects of vagotomy on lipopolysaccharide-induced brain interleukin-1β protein in rats. Auton Neurosci 2000;85:119–126.

18 Cunningham AJ, Murray CA, O'Neill LA, Lynch MA, O'Connor JJ: Interleukin-1β and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. Neurosci Lett 1996;203:17–20.

19 O'Donnell E, Vereker E, Lynch MA: Age-related impairment in LTP is accompanied by enhanced activity of stress-activated protein kinases: Analysis of underlying mechanisms. Eur J Neurosci 2000;12:345–352.

20 Matsuoka Y, Kitamura Y, Takahashi H, Tooyama I, Kimura H, Gebicke-Haerter PJ, Nomura Y, Taniguchi T: Interferon-γ plus lipopolysaccharide induction of delayed neuronal apoptosis in rat hippocampus. Neurochem Int 1999;34:91–99.

21 Chen H, Marshanskiy V, Qi S, Liu D, Vu D, Tremblay J, Hamet P: Effets de la thérapie VasoCare™ sur la protection rénale contre l'ischémie/reperfusion chez le chien. Méd Sci 1999;15:16.

22 Leon LR, Kozak W, Kluger MJ: Role of IL-10 in inflammation. Studies using cytokine knockout mice. Ann NY Acad Sci 1998;856:69–75.

23 Bluthe RM, Castanon N, Pousset F, Bristow A, Ball C, Lestage J, Michaud B, Kelley KW, Dantzer R: Central injection of IL-10 antagonizes the behavioural effects of lipopolysaccharide in rats. Psychoneuroendocrinology 1999; 24:301–311.

24 Molina-Holgado E, Vela JM, Arevalo-Martin A, Guaza C: LPS/IFNγ cytotoxicity in oligodendroglial cells: Role of nitric oxide and protection by the anti-inflammatory cytokine IL-10. Eur J Neurosci 2001;13:493–502.

25 Kondo S, McKenzie RC, Sauder DN: Interleukin-10 inhibits the elicitation phase of allergic contact hypersensitivity. J Invest Dermatol 1994;103:811–814.